A1

This application is a continuation-in-part of United States application Serial No. 08/522,726, filed September 1, 1995, now pending, and is related to United States application Serial No. 09/523,068, filed on even date herewith, now pending, each of which is incorporated by reference herein in its entirety.

Please replace the paragraph beginning at line 30 of page 4 with the following rewritten paragraph:

A2

Figure 2 presents amino acid (aa) sequences of the first SMRT clone (Genbank accession number U37146; SEQ ID NO:1). The aa sequence presented in parentheses (i.e., residues 1330-1376) is an alternatively spliced insert which is not present in the original two-hybrid clone (C-SMRT, aa 981 to C-terminal end). The proline-rich N-terminal domain (aa 1-160) and the glutamine-rich region (aa 1061-1132), as well as the ERDR and SG regions, are also indicated. The C-terminal region of SMRT (aa 1201 to C-terminal end) shows 48% aa identity to RIP13 (Seol et al., *Molecular Endocrinology* 9:72-85 (1995)). The rest of the sequence of RIP13 shows 22% aa identity to SMRT (aa 819-1200).

Please replace the paragraph beginning at line 9 of page 5 with the following rewritten paragraph:

A3

Figures 3A-3D illustrate mediation of the silencing effect of hRARα and hTRβ by SMRT *in vivo*.

Please replace the paragraph beginning at line 4 of page 6 with the following rewritten paragraph:

A4

Figures 5A and 5B provide alignments of the human SMRT (SEQ ID NO:5) and human N-CoR (SEQ ID NO:11) co-repressors.

Please replace the paragraph beginning at line 20 of page 6 with the following rewritten paragraph:

A5

Figure 6C shows alignment of EcR (amino acids 460-510; SEQ ID NO:14), rTR (amino acids 215-265; SEQ ID NO:15), hRAR (amino acids 227-277; SEQ ID NO:16), and rRev-erbA (amino acids 401-451; SEQ ID NO:17) receptor sequences and the secondary structure in the LBD signature motif region. Conserved residues are marked with asterisks. The mutation 483 (AT) is marked at the top of the corresponding residue.

Please replace the paragraph beginning at line 16 of page 7 with the following rewritten paragraph:

A6

Figure 9 shows sequence comparisons of SMRTER (SEQ ID NOs:18, 21, 32, 35, 38 and 39), SMRT (SEQ ID NOs:20, 23, 28, 33, 36 and 40-44), N-CoR (SEQ ID NOs:19, 22, 27, 34, 37 and 45-51), and other related proteins (SEQ ID NOs:24-26, 29-31 and 52). The SANT domains of various proteins are listed (SEQ ID NOs:21-31). Percentages of identity/similarity compared to SMRTER are shown on the right. Two potential helices are predicted in the N-terminal half of the SANT domain. Black boxes indicate identical sequences; gray boxes indicate similar or partially identical sequences.

Please replace the paragraph beginning at line 16 of page 8 with the following rewritten paragraph:

A7

Figure 12C shows an alignment of SMRD3 of SMRTER (amino acids 2564-2588 of SEQ ID NO:12) and an mSin3-interacting domain of N-CoR (amino acids 1835-1859 of SEQ ID NO:11). Conserved residues are boxed. An asterisk indicates the region where the mutation (Gly) was generated. Minus signs indicate that the interaction between SMRD3 and Sin3A was not detectable in the yeast two-hybrid assays. Repression was measured by comparing the transcriptional activity of Gal4-SMRD3 M2 or Gal4-SMRD3 M3 to that of wild-type Gal4-SMRD3 using transfection experiments as described above.

Please replace the paragraph beginning at line 4 of page 42 with the following rewritten paragraph:

A8

The function of SMRT as a silencing mediator (co-repressor) of RAR and TR is analogous to mSin3 in the Mad-Max-Sin3 ternary complex (Schreiber-Agus et al., *Cell* 80:777-786 (1995); and Ayer et al., *Cell* 80:767-776 (1995)). Because GAL-SMRT functions as a potent repressor when bound to DNA, it is reasonable to speculate that the function of the unliganded receptors is to bring with them SMRT to the template via protein-protein interaction. Thus, the repressor function is intrinsic to SMRT as opposed to the TR or RAR itself (Baniahmad et al., *Proc. Natl. Acad. Sci. USA* 90:8832-8836 (1993); and Fondell et al., *Genes Dev* 7:1400-1410 (1993)). It is demonstrated herein that the ligand triggers a dissociation of SMRT from the receptor, which would lead to an initial step in the activation process. This would be followed (or be coincident) with an induced conformational change in the carboxy-terminal transactivation domain (known as AF-2), allowing association with co-activators on the transcription machinery (Douarin et al., *EMBO J.* 14:2020-2033 (1995); Halachmi et al., *Science* 264:1455-1458 (1994); Lee et al., *Nature* 374:91-94 (1995); and Cavailles et al., *Proc. Natl. Acad. Sci. USA* 91:10009-10013 (1994)). Thus, as has previously been suggested (Damm and Evans, (1993), *supra*), the ligand dependent activation of TR would represent two separable processes including relief

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of repression and net activation. The isolation of SMRT now provides a basis for dissecting the molecular basis of trans-repression.

Please replace the paragraph beginning at line 9 of page 43 with the following rewritten paragraph:

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Total bacteria extracts expressing GST fusions of hRARα (aa 156-462) or hRXRα LBD (aa 228-462) and control extracts expressing GST alone or GST-PML (promyelocytic leukemia) fusion protein were subjected to SDS/PAGE and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, pH 8.3/ 192 mM glycine/ 0.01% SDS). After denaturation/renaturation from 6 M to 0.187 M guanidine hydrochloride in HB buffer (25 mM HEPES, pH 7.7/25 mM NaCl/5 mM $MgCl_2$/1 mM DTT) filters were saturated at 4°C in blocking buffer (5% milk, then 1% milk in HB buffer plus 0.05% NP40). *In vitro* translated ^{35}S-labeled proteins were diluted into H buffer (20 mM Hepes, pH 7.7/75 mM KCl/0.1 mM EDTA/2.5 mM $MgCl_2$/0.05% NP40/ 1% milk/1 mM DTT) and the filters were hybridized overnight at 4°C with (1 μM) or without ligand. After three washes with H buffer, filters were dried and exposed for autoradiography or quantitated by phosphoimager.

Please replace the paragraph beginning at line 25 of page 43 with the following rewritten paragraph:

A10

For yeast two-hybrid screening, a construct expressing the GAL4 DBD-hRXRα LBD (aa 198-462) fusion protein was used to screen a human lymphocyte cDNA library as described (Durfee et al., (1993), *supra*). The first SMRT cDNA (SEQ ID NO:1) was isolated from a human HeLa cDNA library (Clontech) using the two-hybrid insert as a probe.

Please replace the paragraph beginning at line 16 of page 44 with the following rewritten paragraph:

A11

The first SMRT sequence cloned encodes a polypeptide of 1495 amino acids rich in proline and serine residues (see Figure 2 and SEQ ID NO:1). Genbank database comparison reveals similarity of the C-terminal domain of SMRT to a partial cDNA encoding another receptor interacting protein, RIP13 (Seol et al., (1995), *supra*), whose role in receptor signaling is unknown. Within this region, there can be identified several potential heptad repeats which might mediate protein-protein interaction with the "α-helical sandwich" structure (Bourguet et al., *Nature* 375:377-382 (1995)) of the ligand binding domain (LBD) of receptors.

Please replace the paragraph beginning at line 18 of page 48 with the following rewritten paragraph:

A12

In principle, over expression of SMRT should restore repressor activity when co-expressed with v-erbA or RAR403 competitors. Indeed, results presented in Figure 3C show that both the whole first SMRT clone (SEQ ID NO:1) and its C-terminal domain of SMRT (C-SMRT) can titrate out v-erbA or RAR403 competitor activity and re-endow GAL-RAR and GAL-TR with silencing activity. In contrast, neither v-erbA nor SMRT show any effect on the transactivation activity of GAL-VP16 fusion. Thus, SMRT is able to block the titration effect of v-erbA and RAR403 and functionally replaces the putative SMRT co-repressor in this system.

Please replace the paragraph beginning at line 30 of page 48 with the following rewritten paragraph:

A13

If SMRT is the mediator of transcription silencing of TR and RAR by interaction with template-bound unliganded receptors, then direct recruitment of SMRT to a heterologous promoter should result in repression of basal level activity. This was tested by fusing the whole first SMRT clone (SEQ ID NO:1) to the GAL4 DBD (GAL-SMRT). The effect of the resulting fusion protein on the activity of the thymidine kinase promoter containing four GAL4 binding sites was analyzed. Figure 3D shows that GAL-SMRT, like GAL-TR, can silence basal promoter activity in a dose-dependent manner. In contrast, GAL-RXR shows no repression.

Please replace the paragraph beginning at line 19 of page 49 with the following rewritten paragraph:

A14

An examination of the previously described human SMRT co-repressor revealed that the first eight amino acids and upstream sequences were derived from a portion of ribonucleoprotein K sequence. Accordingly, a mouse spleen cDNA lambda ZAP II library (Stratagene; La Jolla CA) was screened at low stringency with a probe corresponding to the approximately 1,000 5' base pairs (bp) of the previously identified human SMRT (s-SMRT; SEQ ID NO:1). A 3.5 kilobase (kb) cDNA fragment was obtained that contained a unique sequence in addition to known s-SMRT sequence. The 5' end of this cDNA, and subsequently obtained clones, was used in successive rounds of screening of the mouse spleen cDNA library and a mouse brain cDNA library (Stratagene) and the full-length SMRTα isoform cDNA (SEQ ID NO: 6) and SMRTβ isoform cDNA (SEQ ID NO: 8) were obtained. The mouse SMRT (m-SMRT) 5' sequence then was used at low stringency to screen a human pituitary cDNA library (Stratagene) to obtain the full-length human SMRT (h-SMRT) cDNA (SEQ ID NO:4). All cDNA clones were sequenced on both strands using standard methods, and have been deposited with GenBank as Accession No. AF113003 (h-SMRT; SEQ ID NOS: 4 and 5); Accession No. AF113001 (m-SMRTα ; SEQ ID NOS: 6 and 7); and Accession No. AF113002 (m-SMRTβ ; SEQ ID NOS: 8 and 9).

Please replace the paragraph beginning at line 6 of page 50 with the following rewritten paragraph:

A15

By sequentially shifting between the mouse spleen and mouse brain cDNA libraries, several clones containing a potential starting methionine and 5' untranslated region sequences were obtained. The complete polypeptide sequences of m-SMRT (SEQ ID NO: 7) and h-SMRT (SEQ ID NO: 5) are provided. In addition, a splice variant isolated from the mouse brain cDNA library (SEQ ID NO:8) encoded an m-SMRT co-repressor (SEQ ID NO:9) containing a deletion of amino acids 36 to 254 of SEQ ID NO: 7. The two m-SMRT co-repressors are designated SMRTα (SEQ ID NO: 7) and SMRTβ (SEQ ID NO: 9). Based on sequence similarity to N-CoR (see below), this deletion in m-SMRTβ removes the majority of the sequence in h-SMRT and m-SMRTα that is homologous to N-CoR repression domain 1 (RD1), including a portion of the Sin3A binding region.

Please replace the paragraph beginning at line 18 of page 50 with the following rewritten paragraph:

A16

The cloned h-SMRT (SEQ ID NO: 4) encodes a polypeptide that contains an additional 1130 amino acids at the amino terminus as compared to the previously described human SMRT co-repressor. The full length h-SMRT shares 84% identity with m-SMRTα. A comparison of h-SMRT (SEQ ID NO: 5) and N-CoR (SEQ ID NO: 11) revealed that the N-terminal extension of h-SMRT (amino acids 1 to 1030) and N-CoR (amino acids 1 to 1031) share approximately 41% identity, which is somewhat higher that the 36% identity shared between the full length proteins. However, regions within the N-CoR and SMRT N-termini share striking homology (Figures 4A and 4B).

Please replace the paragraph beginning at line 19 of page 55 with the following rewritten paragraph:

A17

To investigate whether repression by the ecdysone receptor (EcR) in CV-1 cells is mediated by its association with a vertebrate corepressor and whether such an interaction, if it does occur, is impaired by the A483T mutation, a mammalian two-hybrid assay with Gal4-c-SMRT was conducted.

Please replace the paragraph beginning at line 1 of page 66 with the following rewritten paragraph:

A18

In vitro pull down assays (Example 12) were conducted to determine whether EcR interacts with ERID1 and ERID2. In vitro translated 35S-methionine-labeled EcRB1 alone, or a mixture of 35S-methionine-labeled EcRB1 and unlabeled USP, or 35S-methionine-labeled USP alone, were incubated with GST, GST-ERID1 (amino acids 1698-2063 of SEQ ID NO:12), or GST-ERID2 (amino acids 2929-3038 of SEQ ID NO:12). GST-ERID1 and GST-ERID2, but not GST alone, pull down labeled EcR, whereas little interaction is found between USP and any of the three GST proteins. In addition, the pull-down complex was disrupted by the addition of 3μM MurA when USP is present. These in vitro results establish that SMRTER and EcR may interact directly.

Please replace the paragraph beginning at line 12 of page 66 with the following rewritten paragraph:

A19

Further in vitro tests were conducted to determine if ERID1, ERID2, and c-SMRT compete with each other to bind EcR. Gal4-ERID1 (amino acids 1698-2063 of SEQ ID NO:12) or Gal4-ERID2 (amino acids 2929-3181 of SEQ ID NO:12), along with EcR-vp16 and USP, were transfected in CV-1 cells as described above. In this

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competition experiment, additional ERID1, ERID2, and c-SMRT (Chen et al., (1996), *supra*) were cotransfected into cells. ERID1 (amino acids 1698-2063 of SEQ ID NO:12) and ERID2 (amino acids 2929-3038 of SEQ ID NO:12) were tagged with the nuclear targeting signal (MAPKKKRKV) (SEQ ID NO:13) to ensure that these proteins were localized in nuclei. As shown in Figure 11C, interaction between each Gal4-ERID fusion and EcR-vp16:USP was significantly decreased by both ERIDs and by c-SMRT. Interestingly, a more prominent effect was observed in experiments when Gal4-ERID1 (amino acids 1698-2063 of SEQ ID NO:12) was challenged by ERID2, and, conversely, a more efficient competition was achieved by ERID1 to Gal4-ERID2 (amino acids 2094-3181 of SEQ ID NO:12). Together, these results suggest that ERID1, ERID2, and c-SMRT may bind similar or overlapping surface(s) in EcR.

Please replace the Abstract paragraph on page 76 with the following rewritten Abstract:

A20

The present invention relates to isolated polynucleotides encoding a family of silencing mediators of retinoic acid and thyroid hormone receptor (SMRT) isoforms, including vertebrate and invertebrate isoforms thereof. The invention also relates to polypeptide SMRT co-repressors encoded by invention SMRT polynucleotides, and to peptide portions thereof that can modulate transcriptional potential of a nuclear receptor. In addition, the invention relates to chimeric molecules and to complexes containing a SMRT co-repressor or peptide portion thereof, to antibodies that specifically bind such compositions, and to methods for identifying an agent that modulates the repressor potential of a SMRT co-repressor. The invention also provides methods for identifying an agent that modulates a function of a SMRT co-repressor; for modulating the transcriptional potential of a nuclear receptor in a cell using the compositions of the invention; and for identifying a molecule that interacts specifically with a SMRT co-repressor.